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Effect of antiarrhythmic drugs on small conductance calcium – activated potassium channels

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Abstract

Atrial fibrillation (AF) is the most common type of arrhythmia. Current pharmacological treatment for AF is moderately effective and/or increases the risk of serious ventricular adverse effects. To avoid ventricular adverse effects, a new target has been considered, the small conductance calcium-activated K^+ channels ($K_{Ca2.X}$, SK channels). In the heart, $K_{Ca2.X}$ channels are functionally more important in atria compared to ventricles, and pharmacological inhibition of the channel confers atrial selective prolongation of the cardiac action potential and converts AF to sinus rhythm in animal models of AF. Whether antiarrhythmic drugs (AADs) recommended for treating AF target $K_{Ca2.X}$ channels is unknown. To this end, we tested a large number of AADs on the human $K_{Ca2.2}$ and $K_{Ca2.3}$ channels to assess their effect on this new target using automated whole-cell patch clamp. Of the AADs recommended for treatment of AF only dofetilide and propafenone inhibited $hK_{Ca2.X}$ channels, with no subtype selectivity. The calculated IC_{50} were $90 \pm 10 \mu\text{mol/l}$ vs $60 \pm 10 \mu\text{mol/l}$ for dofetilide and $42 \pm 4 \mu\text{mol/l}$ vs $80 \pm 20 \mu\text{mol/l}$ for propafenone ($hK_{Ca2.3}$ vs $hK_{Ca2.2}$). Whether this inhibition has clinical importance for their antiarrhythmic effect is unlikely, as the calculated IC_{50} values are very high compared to the effective free therapeutic plasma concentration of the drugs when used for AF treatment, 40,000-fold for dofetilide and 140-fold higher for propafenone.

Key words: atrial fibrillation; K_{Ca2} channel; calcium-activated potassium channel; antiarrhythmic drug; automated patch clamp.

Chemical compounds studied in this article

Amiodarone hydrochloride (PubChem CID: 441325), disopyramide (PubChem CID: 3114), dofetilide (PubChem CID: 71329), dronedarone (PubChem CID: 208898), flecainide (PubChem CID: 3356), ibutilide (PubChem CID: 60753), quinidine (PubChem CID: 441074), propafenone (PubChem CID: 4932), sotalol (PubChem CID: 5253) and vernakalant hydrochloride (PubChem CID: 9930048).

1. Introduction

AF has become one of the most important public health problems in developed countries. The total lifetime risks for development of AF are 1 in 4 for 40 years age people and older (Lloyd-Jones et al., 2004). Furthermore, it is expected that by 2030, only in Europe, a total of 14 - 17 million people will have AF and 120,000 - 215,000 new cases will appear every year (Zoni-Berisso et al., 2014). Therefore, it is important to have effective and safe therapeutic strategies to treat patients with AF.

Over the past years, a number of drugs have been developed for rhythm control therapy and treatment of AF (Waks and Zimetbaum, 2016). Unfortunately, these drugs are only moderately effective, have many adverse effects and/or may trigger ventricular arrhythmias. Even recently approved drugs, like vernakalant, have limited effects on the treatment of AF and efficacy of the compound is drastically reduced after just a few days of consistent atrial fibrillation (Tsuji and Dobrev, 2013). Therefore, it is important to find new atrial selective targets for AF treatment.

One promising new target is the small conductance calcium-activated potassium channel ($K_{Ca2.X}$ or SK channel). This ion channel plays an important role on the late repolarization phase of especially atrial cardiomyocytes and its inhibition results in the prolongation of the effective refractory period (Diness et al., 2010), a strategy that has been used for the development of class III antiarrhythmic drugs (Schmitt et al., 2014). Moreover, this protein is functionally more important in atria when compared to ventricles (Xu et al., 2003), which could help avoid possible ventricular adverse effects. It has also been demonstrated that pharmacological inhibition of the $K_{Ca2.X}$ channel confers atrial selective prolongation of cardiac action potential and converts AF to

sinus rhythm in a number of small and large animal models (Haugaard et al., 2015; Qi et al., 2014; Skibsbye et al., 2011).

Whether antiarrhythmic drugs (AAD) recommended for treating AF target $K_{Ca2.X}$ channels is unknown. To this end, we tested a large number of AADs (Table 1) on the human $K_{Ca2.X}$ channels to assess their effect on this new target and its clinical significance.

2. Materials and methods

2.1. Cell culture

Two different stable HEK293 cell lines expressing one specific subtype of the human K_{Ca2} channel, either $K_{Ca2.2}$ or $K_{Ca2.3}$, obtained from NeuroSearch A/S (Ballerup, Denmark) (Strøbæk et al., 2004), were used to conduct the experiments. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM1965, ThermoFisher, USA) supplemented with 2.2 g/l $NaHCO_3$, 5.96 g/l HEPES, 10 ml/l Glutamax (Gibco, USA), 10% foetal bovine serum (Biowest, France), 100 U/ml of penicillin/streptomycin (Sigma, Germany) and 100 μ g/ml geneticin (Gibco, USA) in T175 flasks. On the day of the experiment, when cells were 80-90% confluent, 1 ml of Detachin™ (Amsbio, United Kingdom) was used to detach the cells after being washed with free calcium and magnesium phosphate-buffered saline. Then, the cells were resuspended in 5 ml of serum-free DMEM media containing 0.04 mg/ml soy bean trypsin inhibitor (Sigma, Germany) and 25 mmol/l HEPES buffer (Sigma, Germany).

2.2. Solutions and drugs

Whole cell patch-clamp experiments were conducted using symmetrical K^+ solutions. The extracellular solution contained 0.1 mmol/l $CaCl_2$, 3 mmol/l $MgCl_2$, 150 mmol/l KCl, 10 mmol/l HEPES and 10 mmol/l glucose (pH = 7.4 and 285-295 mOsm) and the intracellular solution contained 8.106 mmol/l $CaCl_2$, 1.167 mmol/l $MgCl_2$, 10 mmol/l EGTA, 150 mmol/l KCl, 10 mmol/l HEPES, 31.25/10 mmol/l KOH/EGTA and 15 mmol/l KOH (pH = 7.2). Final free Ca^{2+} concentration was calculated to 400 nmol/l as previously described (Strobaek et al., 2006). The osmolarity of the intracellular solution was adjusted using sucrose (Sigma, Germany) to match the extracellular solution.

Bicuculline methiodide, propafenone, quinidine, sotalol, flecainide, dofetilide, dronedarone, disopyramide and ibutilide were purchased from Sigma (Germany) and amiodarone hydrochloride was purchased from Tocris (United Kingdom) in their solid forms. The drugs were solubilized in pure dimethyl sulfoxide (Sigma, Germany) at a stock concentration of 10 mmol/l and stored at $-20^{\circ}C$. On the day of the experiment, aliquots were diluted to the desired concentration in the extracellular solution.

Vernakalant was purchased as the pharmaceutical preparation Brinavess 20 mg/ml (Cardiome, United Kingdom) and diluted at the day of the experiment to the desired concentrations in the extracellular solution to conduct the experiments with vernakalant hydrochloride.

2.3. Electrophysiology

Whole-cell experiments were conducted using the automated patch-clamp platform QPatch 16 (Sophion, Denmark). After breaking into the cell and establishing a giga seal, a first application of extracellular solution (saline) was applied. This application was followed by the $K_{Ca2.X}$ inhibitor bicuculline (100 μ mol/l), used as positive control for

functional channel expression. Bicuculline was washed off with two more saline applications before applying the test drug. The last saline application prior to the test drug was used as baseline. After this, the test drug was applied at six increasing concentrations at a time scale that allowed for a potential steady-state in current reduction. Finally, after washing twice with saline, the experiment ended with application of bicuculline (100 $\mu\text{mol/l}$). The different steps of the protocol are illustrated in Fig. 1. Currents were elicited using 200 ms voltage ramps ranging from -80 mV to +80 mV from a holding potential of 0 mV and were applied every 2 s with a sample rate of 10 kHz. To avoid background noise, the recorded signals were filtered using a Bessel filter with a cut-off frequency of 3 kHz. Series resistance compensation was set at 80%.

2.4. Data analysis and statistics

Current amplitudes were measured at -80 mV, and the mean of the last three recordings during each application was used for further analysis. To calculate changes in relative current, the mean values were normalized using the last saline application prior to applying test compound as baseline and the first bicuculline injection as reference for total inhibition of the channel. The data were then plotted and fitted using the following equation on GraphPad Prism 7: $Y = Y_{min} + (Y_{min} + Y_{max}) / (1 + 10^{X - \log IC_{50}})$, where Y is the normalized measured current, X is the logarithm of the dose of the tested drug and IC_{50} the drug concentration needed to inhibit 50% of the current. In all cases a Hill slope of -1.0 was presumed. Individual IC_{50} values were calculated for each experiment and then used to obtain the final mean \pm S.E.M.

Correction for baseline drift was applied if applicable, using the QPatch software and the bicuculline applications at the beginning and the end of the experiment as references.

Student's t-tests were used to determine subtype selectivity of test drugs.

3. Results

3.1. Amiodarone, disopyramide, dronedarone, flecainide, ibutilide, quinidine, sotalol and vernakalant have no effect on the hK_{Ca}2.3 channel.

Currents were recorded from HEK cells stably expressing hK_{Ca}2.3 using an automated patch clamp system. In symmetrical intra- and extracellular K⁺ concentrations, hK_{Ca}2.3 currents displayed a characteristic inwardly rectifying current-voltage relationship and were inhibited by bicuculline (Fig. 1). A first screening of the AADs was conducted on the hK_{Ca}2.3 channel. For each drug we tested six different concentrations, starting at 0.1 µmol/l and finishing at 30 µmol/l. In the case of amiodarone the used concentrations were between 0.3 µmol/l and 100 µmol/l (Fig. 2).

Amiodarone (n = 6), disopyramide (n = 8), dronedarone (n = 5), flecainide (n = 6), ibutilide (n = 5), quinidine (n = 5), sotalol (n = 5) and vernakalant (n = 5) had no effect on the hK_{Ca}2.3 current (Fig. 2). Only dofetilide and propafenone showed concentration-dependent inhibition at the highest concentrations on the hK_{Ca}2.3 and therefore were selected for further investigation.

3.2. Dofetilide and propafenone are weak inhibitors of both hK_{Ca}2.3 and hK_{Ca}2.2 with no subtype selectivity.

In order to obtain a complete inhibition curve for dofetilide and propafenone, a second round of experiments was conducted using higher concentrations, starting at 1 $\mu\text{mol/l}$ and finishing at 300 $\mu\text{mol/l}$ (Fig. 3).

As can be seen in Fig. 3A and Fig. 3B, dofetilide and propafenone inhibited the $\text{hK}_{\text{Ca}2.3}$ current in a dose dependent manner. The effect started at 10 $\mu\text{mol/l}$ for both drugs and reached nearly complete inhibition at 300 $\mu\text{mol/l}$. In the case of propafenone, a pronounced drift of the current was observed at the beginning of each new dose application, even after baseline correction. This drift, which was specific for propafenone, stabilized at the end of each application and it was less pronounced as the concentration of the drug was increased. The calculated IC_{50} for dofetilide and propafenone were $90 \pm 10 \mu\text{mol/l}$ ($n = 13$) and $42 \pm 4 \mu\text{mol/l}$ ($n = 8$) for $\text{hK}_{\text{Ca}2.3}$, respectively (Fig. 4). To address if dofetilide or propafenone displayed subtype selective effects, we also tested both drugs on HEK cells stably expressing the $\text{hK}_{\text{Ca}2.2}$ channel. Dofetilide and propafenone were applied on the $\text{hK}_{\text{Ca}2.2}$ expressing cells at the same concentrations and using the same protocol as described for $\text{hK}_{\text{Ca}2.3}$ channel. Both drugs were also able to inhibit $\text{hK}_{\text{Ca}2.2}$ currents in a concentration dependent manner (data not shown). In this case, the effect of dofetilide started at a lower dose of 3 $\mu\text{mol/l}$. Inhibition by propafenone was first observed at 10 $\mu\text{mol/l}$, as seen in $\text{hK}_{\text{Ca}2.3}$. On the $\text{hK}_{\text{Ca}2.2}$ channel the characteristic drift of propafenone was also present at the beginning of every dose application and it stabilized as the dose of the drug was increased. The calculated IC_{50} for dofetilide and propafenone on the $\text{hK}_{\text{Ca}2.2}$ channel were $60 \pm 10 \mu\text{mol/l}$ ($n = 13$) and $80 \pm 20 \mu\text{mol/l}$ ($n = 6$), respectively. Neither dofetilide ($p = 0.1915$) nor propafenone ($p = 0.1816$) showed any subtype selectivity. Due to the apparent weak binding and fast k_{on} and k_{off} of dofetilide and propafenone

(Fig. 4), only current measurements corresponding to steady states, were considered to calculate the IC_{50} .

4. Discussion

Despite being the most common type of arrhythmia, safe and effective AF treatment continues to be an unmet medical need. It is therefore important to find new strategies and targets to treat this increasingly prevalent disease. It has been revealed that both chronic (Voigt et al., 2012) and paroxysmal AF (Voigt et al., 2014) are associated with abnormal Ca^{2+} -handling, like spontaneous sarcoplasmic reticulum Ca^{2+} -release and delayed after depolarizations. These events may modulate and activate other ion channels, such as $K_{Ca2.x}$, resulting in shorter APDs and increased AF vulnerability. Therefore, the $K_{Ca2.X}$ channel is a promising new atrial selective target whose inhibition has proven to be efficacious in both cardioversion and prevention of new AF episodes in a number of animal models (Goldin Diness et al., 2011; Haugaard et al., 2015; Qi et al., 2014; Skibsbbye et al., 2011). The identification and development of novel drugs targeting the cardiac $K_{Ca2.X}$ channels and the understanding of their role in cardiac function may end up in a whole new AF therapy. AADs recommended for treating AF target a number of ion channels and receptors, but whether they can inhibit $K_{Ca2.X}$ channels has not been systematically tested until now. Amiodarone has previously been reported to inhibit $K_{Ca2.2}$ channels expressed in HEK-293 cells (Turker et al., 2013), but this study is the first to test a large number of the recommended AADs for treatment of AF on the hK_{Ca2} channel. Ten AADs at six different concentrations ranging between 0.1 – 30 $\mu\text{mol/l}$ on the $hK_{Ca2.3}$ channel were tested.

From this first screening, only dofetilide and propafenone showed concentration-dependent inhibition of the hK_{Ca}2.3 current. Amiodarone, disopyramide, dronedarone, flecainide, ibutilide, quinidine, sotalol and vernakalant had no effect on the hK_{Ca}2.3 current at any of the tested concentrations. The study published by Turker et al (2013) characterized the effect of amiodarone on the hK_{Ca}2.2 channel and showed that amiodarone was able to inhibit the I_{KCa} current with an IC₅₀ of 3 µmol/l when intracellular free Ca²⁺ concentration was 1 µmol/l. Our experiments failed to show any effect of amiodarone at any of the tested concentrations on any of the K_{Ca}2 subtypes (data not shown for hK_{Ca}2.2). This contradiction might be explained by the strong calcium dependent effect of amiodarone on the hK_{Ca}2 channel, which was also reported in Turker's work. According to the mentioned study, 30 µmol/l amiodarone were able to inhibit around 80% of the I_{KCa} when the intracellular Ca²⁺ concentration was 1 µmol/l, but the effect dropped drastically when Ca²⁺ concentration was decreased. As they state in the discussion of their work, it is possible that 400 nmol/l of intracellular Ca²⁺ concentration (Desk and Williams, 1994), the one chosen for our screening, is not enough to assess the inhibitory effect of amiodarone at 10 µmol/l, although we were not able to see any significant effect at higher concentrations neither. Another important difference between Turker's work and the present study are the K⁺ concentrations used for the extracellular and intracellular solutions. While Turker used physiological K⁺ concentrations for their experiments, in this study, we used symmetrical K⁺ solutions. In any case, it is improbable that the antiarrhythmic effect of amiodarone is partly due to its hK_{Ca}2 inhibition because, in a clinical situation, the effective free therapeutic plasma concentration of amiodarone for AF treatment is below 1 nmol/l (see Table 1), 3,000 times lower than the previously reported IC₅₀.

The second round of experiments included dofetilide and propafenone. The selected AADs were tested at a higher concentration range (1 – 300 $\mu\text{mol/l}$) in order to obtain complete inhibition curves. Moreover, the same experiments were conducted on both hK_{Ca}2.3 and hK_{Ca}2.2 to assess possible subtype selectivity. The calculated IC₅₀ for dofetilide and propafenone were $60 \pm 10 \mu\text{mol/l}$ and $80 \pm 20 \mu\text{mol/l}$ for hK_{Ca}2.2, and $90 \pm 10 \mu\text{mol/l}$ and $42 \pm 4 \mu\text{mol/l}$ for hK_{Ca}2.3, respectively. Neither dofetilide nor propafenone showed any subtype selectivity. In the case of propafenone, a rapid “run up” of current was observed shortly after the application of the drug in all the performed experiments. This run up stabilizes with time and is less pronounced when the concentration of propafenone is increased. It could be speculated that this phenomenon might be explained by an extremely fast k_{on} and k_{off} of propafenone on the hK_{Ca}2 channel. The fast binding of propafenone on the channel is followed by the also rapid unbinding of the drug, which is depicted as a current run up on the current-time plots, until equilibrium is reached and the current stabilized. The higher the concentration, the faster the equilibrium was reached, reducing the run up of the current. For this reason, only the measurements corresponding to the steady state were considered in order to calculate the IC₅₀.

Although dofetilide and propafenone were able to inhibit both hK_{Ca}2.2 and hK_{Ca}2.3 currents, it is highly improbable that this effect has any importance for their antiarrhythmic effects observed in the clinic. The calculated IC₅₀ for both drugs are extremely high when compared to the highest effective therapeutic free plasma concentration used for AF treatment (see Table 1), 40,000-fold and 140-fold higher for dofetilide and propafenone, respectively.

5. Conclusions

Ten of the most common AADs used for the treatment of AF have been screened to assess their effect on the hK_{Ca}2 channel using whole-cell automated patch-clamp. Only two of them, dofetilide and propafenone, were able to weakly inhibit both hK_{Ca}2.2 and hK_{Ca}2.3 in a concentration-dependent fashion with no subtype selectivity. Since the IC₅₀ of the drugs on the hK_{Ca}2 channel is very high when compared to their effective therapeutic free plasma concentration, it is unlikely that this inhibition has any clinical relevance for their antiarrhythmic effect. More efforts on the development of new effective and safe drugs selectively targeting the K_{Ca}2.X channels should be considered to improve AF treatment.

5.1. Limitations of the study

Manual patch clamp is the gold standard for electrophysiology. However automated patch clamp allows for higher throughput. This technique is successfully and continuously being used for primary and secondary drug screening on a great range of ion channels, including calcium-activated potassium channel (Coleman et al., 2014). Moreover, experiments on automated patch clamp systems are highly reproducible and comparable to the results obtained by the manual patch clamp technique (Korsgaard et al., 2009).

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Figure captions

Fig. 1. Representative current-time plot showing the steps included in the screening protocol. The first reference application (100 $\mu\text{mol/l}$ bicuculline) was used to normalize current inhibition. The last saline application prior to the first application of the drug was used as baseline. Only measurements corresponding to steady states were considered to calculate IC_{50} .

Fig. 2. Voltage ramp protocol used to measure the I_{KCa} currents (top left).

Representative current-voltage graph (top right) showing the measured I_{KCa} current (grey line) and its inhibition by 100 $\mu\text{mol/l}$ bicuculline (black line). Effect of amiodarone ($n = 6$), disopyramide ($n = 8$), dronedarone ($n = 5$), flecainide ($n = 6$), ibutilide ($n = 5$), quinidine ($n = 5$), sotalol ($n = 5$) and vernakalant ($n = 5$) on $\text{hK}_{\text{Ca}2.3}$ (mean \pm S.E.M.). Increasing concentrations of AADs were tested on $\text{hK}_{\text{Ca}2.3}$, starting from 0.1 $\mu\text{mol/l}$ and up to 30 $\mu\text{mol/l}$. In the case of amiodarone, the starting concentration was 0.3 $\mu\text{mol/l}$ and the highest concentration 100 $\mu\text{mol/l}$.

Fig. 3. Representative current-time plots of A) dofetilide and B) propafenone inhibition on the $\text{hK}_{\text{Ca}2.3}$ (left) and their current-voltage recordings (right).

Fig. 4. Inhibition curves of dofetilide and propafenone on $\text{hK}_{\text{Ca}2.2}$ (grey lines) and $\text{hK}_{\text{Ca}2.3}$ (black lines). None of the compounds showed any subtype selectivity.

Table 1 list of tested AADs, current or channels/receptors blocked by the drug and their effective free therapeutic plasma concentration for AF treatment.

Drug	Current/channel/receptors (Waks and Zimetbaum, 2016)	Effective free therapeutic plasma concentration (nmol/l)
Amiodarone	I_{Na} , I_{Kr} , I_{Ks} , I_{Kur} , I_{CaL} , I_{KAch} , I_{to} , α , β	0.3 - 0.6 (Redfern et al., 2003)
Disopyramide	I_{Kr} , I_{Na} , I_{KAch}	215 - 800 (Redfern et al., 2003)
Dofetilide	I_{Kr}	0.4 - 1.5 (Redfern et al., 2003)
Dronedarone	I_{Na} , I_{Kr} , I_{Ks} , I_{K1} , I_{CaL} , I_{KAch} , α , β	150 – 300 ¹ (Patel et al., 2009)
Flecainide	I_{Na}	400 – 900 (Redfern et al., 2003)
Ibutilide	I_{Kr}	0.8 - 150 (Redfern et al., 2003)
Propafenone	I_{Na} , β	30 - 300 (Redfern et al., 2003)
Quinidine	I_{Kr} , I_{Ks} , I_{to} , I_{KATP} , I_{CaL} , I_{Na} , I_{KAch}	750 – 3,000 (Redfern et al., 2003)
Sotalol	I_{Kr} , β	2,000 – 15,000 (Redfern et al., 2003)
Vernakalant	I_{Kur}	1,000 – 10,000 (Fedida, 2007)

¹steady state plasma concentration after 7 days.

Figure 1

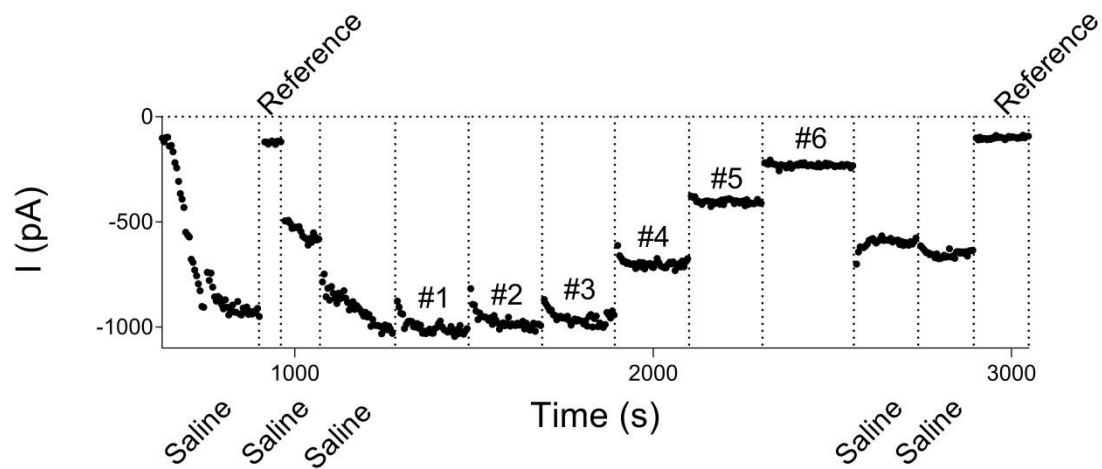


Figure 2

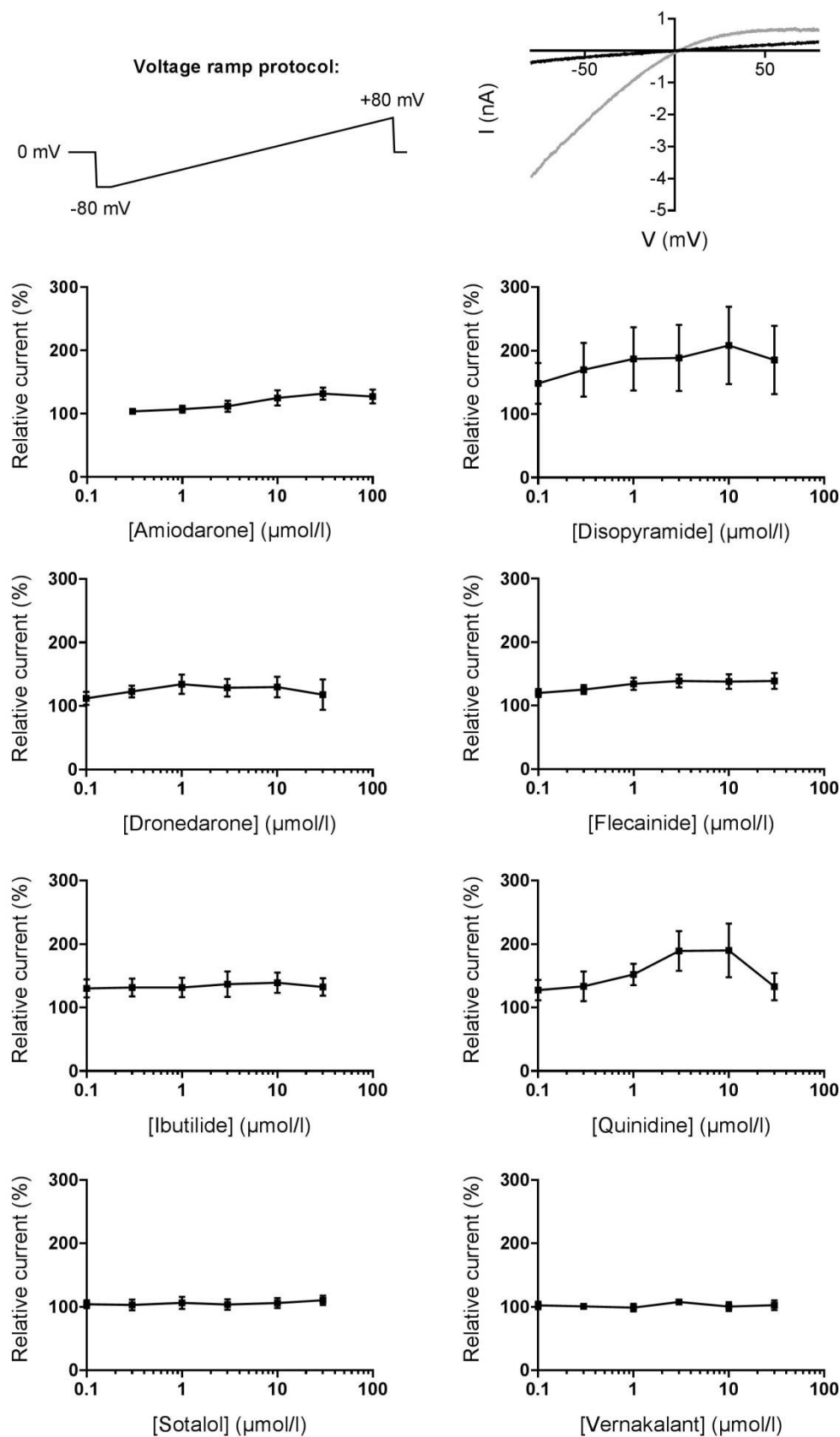


Figure 3

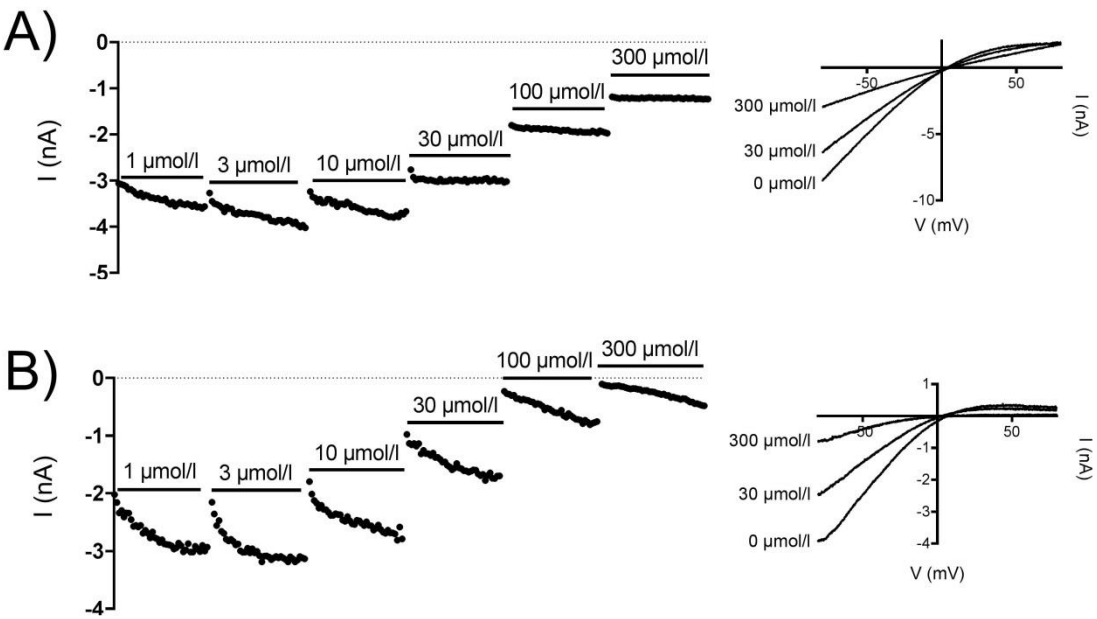


Figure 4

